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(54) Title: DETACHMENT OF ANCHORAGE-DEPENDENT CELLS FROM MICROCARRIERS

(57) Abstract

A method for detaching cells from positively-charged cell culture microcarriers. In this method microcarriers laden with attached cells are exposed to a proteolytic agent at relatively high pH and then subjected to mild shear.

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DETACHMENT OF ANCHORAGE-DEPENDENT CELLS FROM MICROCARRIERS

Description

Field of the Invention

This invention is in the field of cell biology and pertains to a method of detaching anchorage-dependent cells from positively charged microcarriers or other positively-charged substrates.

Background of the Invention

Many types of mammalian cells used for the production of biologicals are anchorage-dependent, that is, they grow only when they can attach themselves to a surface. Indeed, most normal mammalian cells are anchorage-dependent. Conventionally, anchorage-dependent cells are grown in small flasks or in roller bottles - cylindrical vessels that are oriented with their long axis horizontal and are continuously rotated. However, these vessels are not generally suitable for large-scale processes. Alternative methods for cell culturing have been proposed, including plastic bags, stacked plates, spiral films, glass bead propagators, artificial capillaries, and microcarriers.

The concept of using microscopically small beads called microcarriers to enlarge the surface area in a single bioreactor for attachment and growth of anchorage-dependent cells was suggested by van Wezel. See van Wezel et al., (1967) "Growth of Cell Strains and Primary Cells on Microcarriers in Homogenous Culture" Nature 216, 64-65. An example

of microcarriers is a positively-charged bead measuring about 50 to several hundred microns in diameter and typically composed of diethylaminoethyl (DEAE)-substituted dextran.

Microcarrier systems for cell cultivation offer certain advantages over other large-scale cell propagation methods. For one, a much higher ratio of growth surface to vessel volume (S/V) can be obtained with microcarriers in comparison to both traditional and newly developed alternative techniques. The increase in S/V attainable allows the construction of a single-unit homogeneous or quasi-homogeneous batch or semi-batch propagator for high volumetric productivity. Thus, a single stirred tank vessel with simple feedback control for pH and pO_2 presents a homogeneous environment for a large number of cells and eliminates the need for expensive and space consuming, controlled environment incubators. Also, the total number of operations required per unit of cells produced is drastically reduced. As a result, microcarriers offer economies of capital, space and manpower in the production of anchorage-dependent cells, relative to previous production methods.

Microcarriers also offer the advantage of environmental continuity because the cells are grown in a single controlled environment. They provide the potential for growing anchorage-dependent mammalian cells under one set of environmental conditions which can be regulated to provide constant, optimal cell growth.

To exploit microcarrier technology, it is necessary to serially propagate cells entirely on microcarriers, from initial inoculum through scale-up to final harvest of the cells or cell products. Use of microcarriers, however, generally has been limited to the final stage of the scale-up procedure. The principal obstacle to their use has been the difficulty in detaching cells from the microcarriers in a viable condition, which is a prerequisite if the culture is to serve as an inoculum for a subsequent larger-scale batch.

Because most anchorage-dependent cells will not spontaneously migrate from bead to bead, to achieve transfer, cells must be detached from the beads, dispersed in the medium, and then allowed to reattach to a larger surface area provided by a larger number of beads. Techniques developed to detach cells anchored to traditional substrates such as the walls of glass or plastic culture vessels have proven inadequate for most cells growing on microcarriers. One standard technique, treating the cells with trypsin, a proteolytic enzyme, followed by mechanically shearing the cells off the surface (see Waymouth in The Growth Requirements of Vertebrate Cells in Vitro, Cambridge Univ. Press, N.Y., p. 118, hereby incorporated by reference), does not separate most anchorage-dependent cells from microcarriers without considerable cell damage and loss. Likewise, detachment by mechanical shear alone (such as repeated passage through a pipette) causes extensive cell mortality.

Other detachment techniques have been attempted, but with limited success. Low frequency ultrasound detaches cells, but also lyses them. Treating cell-ladened dextran-based microcarriers with dextranase to dissolve the beads, leaving unattached cells, has not worked well in practice, and has the added disadvantage of not permitting recycling of the microcarrier beads.

Crespi and Thilly, Biotechnology and Bioengineering 23, 983-993 (1981) have shown that when the anchorage-dependent cell line LLC-MK_2 and the non-anchorage-dependent CHO-K1 cell line are grown in media with a reduced calcium concentration, continuous subculture is possible by the simple expedient of periodically adding fresh microcarriers suspended in fresh medium. The lower calcium concentration apparently affects the cell-microcarrier bond, permitting a bead-to-bead transfer that does not occur at normal calcium concentrations. This technique is limited, however, to those cell lines both capable of growing in a reduced calcium medium and susceptible to the reduced-calcium effect on adherence. Many other cell lines, including human and chick fibroblasts, require higher levels of calcium for optimal growth, and so the technique of Crespi and Thilly may not have widespread utility.

Another method has been suggested by Joseph Feder and William R. Tolbert, Scientific American 248, 36-43 (1983), who found that if cell-laden microcarriers were allowed to settle and aggregate,

the cells' adhesion to the microcarriers became weakened, and a brief treatment with trypsin released the cells from the microcarriers. This technique has not found general acceptance because the cells may suffer significant loss due to limited access to fresh medium during the settling and aggregation step. Furthermore, when cells are allowed to aggregate, it is usually difficult to disperse them evenly, making scale-up difficult.

Summary of the Invention

This invention constitutes a method of detaching cells from positively-charged cell culture microcarriers such as DEAE-substituted polydextran beads or from other positively-charged substrates. Microcarriers laden with attached cells are exposed to a proteolytic agent(s), such as the enzyme trypsin, at a relatively high pH, that is, a pH of about 7.8 to about 10.0, a pH of about 8.2 being preferred. After proteolytic treatment at high pH, the cell-laden microcarriers are subjected to mild shear by, for example, passage through a glass bead column. Typically more than 90% of the cells are released in a viable state by this treatment. The released cells can be inoculated into a new culture vessel with additional microcarriers and fresh medium and propagated further. Cell growth and cell-product formation is unimpaired by the detachment process.

Brief Description of the Drawings

Figure 1 illustrates the morphological changes

in FS-4 cells brought about by trypsinization at high pH of the cells attached to microcarriers.

Figure 2 illustrates the effect of pH on cell detachment from microcarriers by trypsinization.

Figure 3 illustrates the kinetics of FS-4 cell growth in microcarrier culture. The arrows indicate the time of high pH trypsinization and inoculation into a new culture.

Figure 4 illustrates the effect of pH on the proteolytic activity of trypsin.

Figure 5 illustrates the serial propagation of FS-4 cells on microcarriers of a selected diameter. The arrow indicates time of high pH trypsinization and inoculation into a fresh culture.

Figure 6 illustrates the serial propagation of Vero cells on microcarriers.

Figure 7 illustrates vesicular stomatitis virus production by Vero cells serially propagated on microcarriers using the high pH trypsinization method of cell detachment.

Best Mode of Carrying Out the Invention

In order to achieve serial propagation of cells in microcarrier culture systems, cells are transferred from cell-covered microcarriers to new bare microcarriers. Cells grown on conventional surfaces, such as Petri dishes or roller bottles, can be detached by treatment with a proteolytic agent, most often trypsin, along with mild mechanical perturbation. These cells are then used as the

inoculum of another culture. This method of detachment, however, is ill-suited for detaching most types of anchorage-dependent cells from microcarriers. As a consequence, cells grown in microcarrier culture generally cannot be used to inoculate another culture. Instead, cells grown in roller bottles are required for the inoculation of a microcarrier culture. Because in most instances, a large inoculum concentration is required to achieve desirable growth rates and growth extents in microcarrier systems, a large number of roller bottles is required for the inoculation of a single microcarrier culture. Unfortunately, this makes inoculum preparation a difficult task, especially in large scale operations.

This invention provides a method of detaching cells from positively-charged substrates, such as microcarriers. The method permits direct inoculation of large microcarrier cultures from a seed microcarrier culture and consequently, eliminates the laborious task of preparing large number of roller bottle cultures for inoculation. It permits serial cultivation of anchorage-dependent cells, from seed-culture to larger-scale productive cultures, entirely on microcarriers (or on other positively-charged substrates).

According to the method of this invention, cells can be detached from positively-charged microcarriers in viable condition by treating the cell-laden microcarriers with a proteolytic agent at a pH above the pH of normal culture conditions, that

is, at a pH above about 7.8 to about 10.0, and then generating a shear force between the microcarrier and the cells to remove the cells from the microcarrier. Anchorage-dependent cells detached in this manner remain viable and transfer to and proliferate on fresh microcarriers.

Initial or seed microcarrier cultures of anchorage-dependent cells can be established by standard techniques. See e.g., Levine, D. W., Wang, D.I.C., and Thilly, W. G. (1979) Biotechnol. Bioeng. 21, 821-845. Cells can be detached from microcarriers in seed culture by the method of this invention when they reach any desired growth stage. Cells are usually detached when they reach confluence.

To prepare for the detachment process, cell-laden microcarriers are separated from the growth medium. Any suitable method of separation may be used. For example, the cell-laden microcarriers may be allowed to settle in the culture vessel and the medium supernatant removed by decanting or by suction. Alternatively all or a portion of the medium containing suspended cell-laden microcarriers can be transferred from the growth vessel to a separate vessel for separation.

Before proteolytic treatment, the cell-laden microcarriers should be washed to remove any residual medium. When the cells have been grown in serum-supplemented medium, cell-laden microcarriers must be washed thoroughly because serum will inhibit the activity of the proteolytic agents and interferes with the detachment process. The cells may be

washed with an aqueous isotonic buffer, such as phosphate buffered saline (PBS) or HEPES.

The cell-laden microcarriers are then treated with a proteolytic agent at pH 7.8-10.0. To accomplish this, the settled microcarriers may be resuspended in an aqueous isotonic buffer containing the proteolytic agent, adjusted to the selected pH within the operative range. The optimum pH within the prescribed range is dependent upon a host of factors including the particular proteolytic agent, the type of microcarrier, the type of cell and the conditions of culture and can be determined for any combination of these factors by routine experimentation.

Generally, proteolytic treatment of about 10-15 minutes duration is sufficient to obtain a high degree of detachment.

A chelating agent such as ethylenediamine tetraacetic acid (EDTA) may be added to the solution of the proteolytic agent. The chelating agent acts as a scavenger of divalent cations such as calcium and magnesium. Divalent cations are thought to play a role in cell attachment to substrates, and their removal may aid in detachment.

Suitable proteolytic agents are the proteases trypsin, pronase, collagenase and proteinase K. Mixtures of two or more of these enzymes may be used. The concentration of the proteolytic agent(s) should be that which yields a high degree of detachment with minimal loss of cell viability.

The preferred proteolytic agent is trypsin. Various trypsin preparations are available commercially. A frequently-used trypsin preparation is Bacto-Difco 1:250. A concentration of about 0.1-0.2% (0.1-0.2 g trypsin preparation per 100 ml buffer solution) can be used for the detachment process. FS-4 cells grown on DEAE-dextran microcarriers are detached readily by a 0.2% solution of this trypsin preparation at pH between 8.2 and 9.0; the detached cells reattach to and grow on fresh microcarriers without significant loss of viability. For FS-4 cells, pH 8.2 can be used routinely and provide satisfactory results.

Another suitable trypsin preparation is twice recrystallized trypsin supplied by Worthington Diagnostics. A 1:500 dilution of a 4.0% stock solution of this trypsin preparation yields excellent detachment.

Temperature is a factor which may affect proteolysis at elevated pH. For example, it is known that the temperature at which cells are trypsinized can affect viability during subculturing. McKeehan et al. reported that cells trypsinized at low temperature (4°C) showed a significant improvement in subsequent clonal growth. McKeehan, W. L. et al. "The Use of Low-Temperature Subculturing and Culture Surface Coated with Basic Polymers to Reduce the Requirement for Serum Macromolecules" In: The Growth Requirements of Vertebrate Cells In Vitro, pp. 118-150, Cambridge University Press, 1981. FS-4 cells are detached and successfully cultured after

high pH trypsinization at room temperature. However, for cells which are sensitive to pH, the combination of high pH, low temperature and, possibly, a reduced trypsin concentration may improve cell viability. The most effective combination of parameters can be determined for any particular type of anchorage-dependent cell by routine experimentation.

Additional factors which influence cell detachment by this process include the composition of the growth medium, the concentration of serum supplement, the degree of confluency of the cells at the time of detachment and the length of time that the cells have been in a confluent stage. As these conditions vary, concentration of proteolytic agent, temperature, the concentration of any chelating agent and the pH can be adjusted to provide optimum detachment and cell viability.

After proteolysis at elevated pH, the cells are detached from the microcarrier by application of a gentle shear force. A shear force can be generated in a number of ways. For example, sufficient shear force may be provided by simply pipetting, repeatedly if necessary, a reconstituted suspension of the treated microcarriers. For large cultures, a convenient way of providing effective shear is to pass the treated microcarriers through a column packed with glass beads. Glass beads of about 3 mm in diameter are suitable; column dimensions may vary depending upon the amount of microcarriers. A shear force is generated during passage of the

microcarriers through the column which is sufficient for detachment of cells. The column effluent containing microcarriers and detached cells is collected and can be used directly as an inoculant for a fresh culture.

The method of this invention is believed to be useful for detaching anchorage-dependent cells from any positively-charged substrate suitable for their attachment. Examples of such substrates may be fibers, plates or beads.

Typical microcarriers are comprised of porous beads having a positively charged surface. The beads are made of hydroxyl-containing polymers such as dextran, dextrin, starch, cellulose, polyglucose and substituted derivatives of these polymers. The hydroxyl groups provide sites for attachment of a charge-supplying group which most often are tertiary amines. Some examples of commercially available positively charged microcarriers are ion exchange resins sold under the trade names DEAE-Sephadex A50, and DEAE-Sephadex A25 by Pharmacia. These microcarriers comprise dextran polymers substituted with diethlyaminoethyl (DEAE) groups, generally designated DEAE-substituted dextran beads.

Recently, Levine et al. discovered that the amount of positive charge capacity on a microcarrier can to be controlled within a certain range to result in good growth of a wide variety of anchoragedependent cells. See e.g., United States Patent No. 4,189,534. The range is about 1.0-2.8 mequiv/gram dry, untreated dextran.

DEAE-substituted dextran beads having charge capacity within the range are sold under the trade name Superbeads by Flow General, Inc. Microcarriers of predefined charge capacity can be made by controlling the amount of the charge-supplying moiety substituted on the polymer.

Employing the detachment method of this invention, human diploid fibroblasts (FS-4 strain) were serially propagated on DEAE-dextran microcarriers. Cells were grown to confluence on microcarriers in initial seed culture, then washed several times with at least 30 bead-volumes of phosphate buffered saline (PBS). The beads were then suspended in five volumes of trypsin solution in PBS. In early experiments, the pH of the trypsin solution ranged from about 8.9 to 9.0. In later experiments, it was found that the pH can be reduced to 8.2. After resuspending cell and microcarriers in trypsin solution, the microcarriers were allowed to settle and drain in a glass column or a sintered glass funnel. Trypsinization was allowed to proceed for 5 to 10 minutes. Then the beads were quickly resuspended in fresh medium and passed through a 30 x 1.5 cm column packed with 3 mm glass beads (bed height 20 cm). Typically, more than 90% of the cells on the microcarriers were released. If a significant portion of cells remained attached, the bead suspension was passed through the column for one or more additional times. The cell-microcarrier mixture was then inoculated into a new culture vessel containing prewarmed fresh medium containing new microcarriers.

Most cells reattach to the microcarriers within half an hour. These cells then grew exponentially to confluence.

A continuous monkey kidney cell line (Vero) was also cultivated serially on microcarriers using the detachment technique. The detachment technique is believed applicable to the serial cultivation of many anchorage-dependent cells including chicken embryo fibroblast cells as well as cells which can grow both on a surface and in suspension, such as chinese hamster ovary cells and baby hamster kidney cells.

Importantly cells serially propagated on microcarriers using the detachment process of the invention were capable of product formation. For example, serially cultivated FS-4 cells produced Beta-interferon and Vero cells grown in the same manner supported production of vesicular stomatis virus. This indicates that the high pH trypsin treatment does not impair the productive capabilities of cells. Thus, cells can be grown in large scale culture entirely on microcarriers, and the cells can be used for the production of viruses, vaccines, hormones, lymphokines or other cellular growth by-products.

The method is illustrated further by the following exemplification.

Exemplification

Cells and Cell Culture

Human foreskin fibroblasts, FS-4, were obtained from Dr. Jan Vilcek of the New York University, School of Medicine, New York, New York and deposited at the Cell Culture Center, Massachusetts Institute of Technology. Cells were passaged and maintained in one liter roller bottles (490 cm^2) at 37°C. Cells from one confluent roller bottle were used to inoculate four other roller bottles at each passage. The cells were frozen at passage number twelve (24 population doublings) in Dulbecco's Modified Eagle's (DME) Medium supplemented with 10% dimethylsulfoxide (DMSO) and 5% fetal calf serum (FCS). The frozen cells were thawed and propagated in roller bottles and used for experiments between passage number fifteen to twenty. The growth medium for newly passaged cells was DME supplemented with 5% FCS and 5% calf serum (CS). The composition of DME is shown in Table 1.

Table 1

Composition of Media DME and F-12

Component	DME(mg/L)	F-12(mg/L)
$CaCl_2$	200.0	33.22
$CuSO_4 5H_2O$	---	0.00249
$Fe(NO_3)_3.9H_2O$	0.10	---
$FeSO_4.7H_2O$	---	0.834
KCl	400	224
$MgCl_2$	---	57.2
$MgSO_4$	97.7	---
NaCl	6400	7600
$NaHCO_3$	3700	1176
$NaH_2PO_4.H_2O$	125	---
Na_2HPO_4	---	142
D-Glucose	4500	1802
Hypoxanthine	---	4.10
Linoleic acid	---	0.084
Lipoic acid	---	0.21
Phenol red	15.0	1.20
Putrescine 2HCl	---	0.161
Sodium pyruvate	---	110
Thymidine	---	0.73
L-Alanine	---	8.90
L-Asparagine.H_2O	---	15.0
L-Arginine.HCl	84.0	211
L-Aspartic acid	---	13.3
L-Cystine	48.0	25.0
L-Glutamic acid	---	14.7
L-Glutamine	584	146
Glycine	30.0	7.51
L-Histidine HCl.H_2O	42.0	21.0
L-Isoleucine	105	3.94
L-Leucine	105	13.1
L-Lysine HCl	146	36.5
L-Methionine	30.0	4.48
L-Phenylalanine	66.0	4.96
L-Proline	---	34.5
L-Serine	42.0	10.5
L-Threonine	95.0	11.9
L-Tryptophane	16.0	2.04
L-Tyrosine	72.0	5.40
L-Valine	94.0	11.7
Biotin	---	0.0073
D-Ca pantothenate	4.00	0.480
Choline Chloride	4.00	13.960
Folic acid	4.00	1.30
i-Inositol	7.20	18.0
Nicotinamide	4.00	0.037
Pyridoxal HCl	4.00	0.062
Riboflavin	0.40	0.38
Thiamine HCl	4.00	0.340
Vitamin B_{12}	---	1.36

FS-4 cells were maintained in a roller bottle for up to six weeks after having been inoculated from the previous passage. The medium was replenished weekly with DME supplemented with 2% FCS and 8% newborn calf serum (NCS).

The monkey kidney cell line, Vero, was obtained from Flow Laboratories (McLean, VA). The cultivation conditions were the same as those for FS-4 cells except that the DME medium was supplemented with 10% horse serum (HS) and each confluent roller bottle was used to inoculate eight new roller bottles per passage. All media used were supplemented with penicillin G (100 units/ml) and streptomycin (100 ug/ml).

Staining of Cells for Microscopic Examination

During the cultivation period cell morphology was examined with a microscope regularly. Although this can be performed with unstained cells, the contrast of unstained cells was not sufficient for the preparation of micrographs. To stain cells, 0.2 ml of the cell-laden microcarriers were withdrawn from the culture and placed in a 24-well plate. Fixing and staining were performed at the same time by the addition of 0.05 ml of fixative-staining solution to the sample-containing well. The fixative staining solution contains 40% ethanol and 0.5 g/l of crystal violet. Fixing and staining was allowed to proceed for about one minute before one ml of PBS was added cerefully to dilute the staining solution. The excessive diluted staining solution was decanted by suction and the cells were ready for

microscopic examination and microphotograph preparation.

Preparation of Microcarriers

Microcarriers were prepared by the procedure of Levine et al., Biotechnol. Bioeng. 21, 821-845 (1979) with some modifications. The desired bead size was obtained by sieving Sephadex™ G-50-80 or G-50-150. The microcarriers regularly used in this study were Sephadex™ beads having diameters from 53 to 75 u. Larger Sephadex™ beads having diameters ranging from 90 to 105 u were used in some experiments.

Diethylaminoethylchloride-hydroxychloride (DEAE Cl-HCl; Sigma Chemical Co., St. Louis, MO) was first recrystalized from methylene chloride before being used to prepare microemulsions. To prepare charged microcarriers, 40 g of sieved dry Sephadex beads were suspended in 480 ml of distilled water and placed in a two-liter round-bottom flask. Two hundred and forty ml of 2 M DEAE.HCl were added and the temperature of the reaction mixture was increased to 50°C by rotating the flask in a water bath. After 30 minutes, the reaction was started by the addition of 240 ml of prewarmed 3 N NaOH. The reaction was carried out at 50°C while rotating the round bottom flask in a water bath. The reaction was allowed to proceed for one hour and then quenched by the addition of one liter of distilled water. The beads were poured into two 2-liter sintered glass funnels and were washed with 20 l of

water followed by 12 l of 0.1 N HCl and 24 l of 0.0001 N HCl.

The charge density of microcarriers was quantified immediately after the washings. To measure the charge density, the microcarriers were washed with 10% (w/w) sodium sulfate (75 ml/g Sephadex G-50) and the effluent was collected. The effluent was then titrated with 1.0 N silver nitrate in the presence of potassium chromate indicator. Microcarriers prepared by this procedure typically have an ion exchange capacity of 1.8 to 2.1 meq/g dextran.

To prepare a microcarrier stock suspension, titrated microcarriers were washed with 25 volumes of distilled water followed by 40 volumes of calcium- and magnesium-free phosphate buffered saline (PBS). Washed beads were resuspended in PBS at 10 g/l, autoclaved, and maintained under sterile conditions until use.

Microcarrier Culture

Unless otherwise noted, microcarrier cultures were seeded from growing stock cultures in roller bottles. Two days before inoculation, cells from each roller bottle were trypsinized and then inoculated into two seed roller bottles. All 100 ml culture studies were carried out in 250 ml spinner vessels (Wilbur Scientific Inc., Boston, MA) containing a suspended magnetic impeller (0.8 cm x 4 cm). The impeller was placed 1 cm above the bottom of the flask to avoid destruction of the microcarriers by shear. The spinner flasks were siliconized with 1% Prosil (VWR Sci. Co.) prior to use

to prevent microcarriers from sticking to the vessel wall.

Unless otherwise specified, the medium used for microcarrier culture was Dulbecco's Modified Eagle (DME) medium. For FS-4 cells, the medium was supplemented with 5% fetal calf serum (FCS) and for Vero cells, with 10% horse serum. A microcarrier concentration of 5 g/l was normally used. To prepare a spinner culture, 0.5 g of microcarriers in Phosphate buffered saline (PBS) were allowed to settle and were washed twice with 30 ml of DME. The microcarriers were then reconstituted to 80 ml with serum-supplemented medium and transferred to a spinner flask. The spinner flask was placed in a humidified carbon dioxide incubator to allow equilibration of both temperature and pH before inoculation with cells.

The inoculum was obtained by trypsinizing cells from seed roller bottles with a 0.2% trypsin solution in PBS (no calcium or magnesium) containing 0.02% ethylenediamine tetraacetic acid (EDTA). After trypsinization, cells were pelleted by centrifugation at 800 x g for ten minutes. The pelleted cells were resuspended in the culture medium and the concentration of viable cells was determined by the dye exclusion method using a hematocytometer. The dye employed was 0.1% trypan blue in PBS. The volume of the inoculum was then increased to 20 ml with prewarmed medium and inoculated into the microcarrier suspension. An impeller speed of 65 to 75 rpm was used for agitation.

For the cultivation of FS-4 cells, 50% of the culture medium was removed and replenished with fresh medium on the third day of culture. In experiments of more than six days duration, additional 50% medium changes were performed on the sixth and eighth days. More frequent medium changes were necessary for Vero cells. The first 50% medium change was performed about sixty hours after inoculation and after the fourth day, the medium was changed every day.

For cultivation on microcarriers of larger diameter, the impellers of the spinner were modified. Two 45 degree pitched blades (2.2 x 22 cm) were installed onto the paddle to permit a lower agitation rate (45 rpm).

The 500 ml cultures were carried out in one liter vessels. Eighty cm of silicone rubber tubing (0.058 in. i.d., 0.077 in. in o.d., silastic tubing, Dow Corning) were placed in the vessel. A surface aerator was used for the cultivation of Vero cells to improve oxygen transfer. For FS-4 cells, the dissolved oxygen level could be maintained at about 40% of the saturation level by aeration without the use of silicone rubber tubing.

In DME medium, the pH of the culture ranged from 7.15 to 7.35 for FS-4 cells and from 7.00 to 7.35 for Vero cells. However, when a DME/F12 mixture was used for the FS-4 cells, pH decreased significantly in the absence of pH control. In these experiments, pH was regulated by activating a controller which allowed air to pass through

silicone rubber tubing, thereby reducing the dissolved CO_2 concentration. The air flow rate through the silicone rubber tubing was about 100 ml/min.

Cell growth on microcarriers was monitored according to the method of van Wezel, supra, which is based on the original technique of Sanford et al., J. Natl. Canc. Inst. 11, 772 (1949). A 2 ml sample of the culture was withdrawn from a well-mixed spinner vessel and then centrifuged at 1,000 rpm for 3 minutes. The supernatant was decanted and pelleted microcarriers were resuspended in 2 ml of 0.1% (w/w) crystal violet in 0.1 M citric acid. After incubation for one hour at 37°C, the suspension was mixed with a Pasteur pipette to detach cell nuclei from the microcarriers. Stained nuclei were then counted in a hematocytometer.

Detachment of Cells from Microcarriers

Microcarriers were withdrawn from a spinner flask and placed in a 250-ml plastic centrifuge tube. After the supernatant medium was removed, cells and microcarriers were washed extensively with 30 volumes (ml/ml beads) of PBS. A trypsin solution was prepared by diluting a concentrated stock solution ten-fold in a saline solution containing 30 mM HEPES buffer, 4 mM glucose, 3 mM KCl, 130 NaCl, 1 mM Na_2HOP_4 and 0.0033 mM phenol red (McKeehan et al., 1977). The diluted trypsin solution contained 0.2% trypsin and 0.02% EDTA. The trypsin solution could also be prepared in PBS with pH adjustment. The pH was adjusted with 2 N NaOH to 8.9 to 9.0.

Solutions of pH 8.2 to 8.4 were also used to detach cells successfully.

Trypsinization was performed in a sintered glass funnel (50 ml) or in a chromatographic column (2.5 x 10 cm, Biorad Co., NY) to facilitate the removal of excess trypsin solution. To each 10 ml of washed microcarrier pellet 50 ml of trypsin solution were added. The microcarrier suspension was then transferred to the sintered glass funnel or the chromatographic column, the microcarriers were allowed to settle and the excess trypsin to flow through the bed formed by microcarriers. A portion of the microcarriers was withdrawn periodically for microscopic examination. When cells became more spherical, residual trypsin was suctioned from the top of the microcarrier bed. When a low cell transfer ratio (less than four) was used, residual trypsin was removed by washing the cells and microcarriers with one volume of DME medium supplemented with 5% FCS (or 10% horse serum for Vero cells). When a high multiplication ratio was used, the amount of trypsin carried over to the next cultivation step was small and therefore the last washing with serum-containing medium was omitted. The duration of trypsinization varied from 3 to 10 minutes. In all cases, morphological change was used as the basis for determining the duration of trypsinization.

After trypsinization, microcarriers were suspended in prewarmed medium. Cells so treated could be detached by repeatedly pipetting the medium

if the volume was small. For larger volumes, the microcarrier suspension was passed through a 30 cm high, 1.5 cm diameter column packed with glass beads having a diameter of about 3 mm. The bed height of glass beads was 20 cm. Typically more than 90% of the cells on the microcarriers were released under such conditions. If a significant portion of cells remained attached, the microcarrier suspension was repeatedly passed through the column. The cell suspension, along with the used microcarriers, was then inoculated into a new culture vessel. The inoculation procedure was the same as described above in the microcarrier culture section. Under the conditions used, cells reattached to microcarriers within half an hour after inoculation into the new vessels.

Measurement of Trypsin Activity

The proteolytic activity of trypsin was measured with a chromogenic substrate, azocollagen (Azocoll, Sigma Chemical Co.). Solutions of Azocoll (10 mg/ml) of different pH were prepared in 30 mM buffer. Trypsin solutions of various pH's were prepared in 30 mM HEPES buffer. To measure trypsin activity, 4 ml of the trypsin solution were added to test tubes containing an equal volume of substrate solution at the same pH. The mixtures were incubated at 37°C for 15 minutes. One ml samples were withdrawn from each tube periodically and filtered through filter paper. The color intensity of the filtrate determined with a spectrophotometer (520 nm).

Vesicular Stomatitis Virus (VSV) Production

A 500 ml culture of Vero cells in a one-liter vessel was used for production of VSV as described by Giard et al. (1977), "Virus Production with a Newly Developed Microcarrier System" Appl. Environ, Microbiol. 34, 668-672. The multiplicity of infection (moi), defined as the ratio of viral plaque-forming units (PFU) to cell number, was 0.1. Before the addition of VSV, microcarriers were allowed to settle and 250 ml of the medium were removed to reduce the culture volume. For one hour, the pH was maintained between 6.5 and 6.8, and the culture was agitated occasionally (about one minute in every ten) to ensure even adsorption of virus onto the cells. Subsequently, 250 ml of medium were added to restore the culture volume to 500 ml. The pH was readjusted to 7.3 and continuous agitation resumed. Five ml samples were withdrawn periodically for the titration of the virus. The samples were centrifuged at 2,000 rpm at 4°C for 10 minutes to remove cell debris. The supernatant was frozen at -20°C until virus plaque assays were performed.

Virus Plaque Assay

The VSV titer was determined by a plaque assay, using secondary chicken embryo fibroblasts. Ten-day-old chick embryos were used to establish the primary culture. Two days prior to the plaque assay, confluent primary chicken or embryo fibroblasts were trypsinized and inoculated into 6 cm diameter Petri dishes (5×10^5 cells per dish) to start secondary cultures. The medium for secondary

cultures was DME supplemented with 1% chicken serum, 2% tryptose phosphate broth and 1% calf serum. To perform the plaque assay, serial ten-fold dilutions of the virus were made in DME supplemented with FCS (10%) and .2 ml of diluted sample was inoculated onto each dish. Each sample was assayed in duplicate. The virus was allowed a one-hour adsorption period at 37°C in a humidified 10% CO_2 atmosphere. After the adsorption period, 2 ml of 1% agar overlay (consisting of DME medium + 10% FCS) was added to each dish, and then the dishes were incubated at 37°C for two days. Plaques were scored after the dishes were stained with a 1:2, 500 dilution of neutral red in PBS.

Interferon Production

Interferon was produced according to the procedure of Giard et al., Develop. Indust. Microbiol. 22, 299-309 (1981), a modification of the original procedure of Havell and Vilcek, Antimicrob. Agents Chemotherapy 2, 476-484 (1972). The procedure involves three stages: priming, induction and production.

Priming was carried out at 37°C. FS-4 cells on microcarriers were washed twice with DME without serum and then resuspended in DME medium supplemented with 1% FCS ($1.0 - 1.2 \times 10^6$ cells/ ml.). Cells were primed by addition of human Beta-interferon at a concentration of 50 units/ml; the priming was carried out for sixteen hours.

For induction, cells were washed twice with serum-free DME medium. The induction was carried

out in serum-free DME medium at 34°C. The inducer, poly I.polyC (PL Biochem. Co., Milwaukee, WI), was added at 50 ug/ml together with 10 ug/ml of cycloheximide (Sigma Chem. Co.). After 4 hours of incubation at 34°C, actinomycin D (Sigma Chem. Co.) was added to give a concentration of 1 ug/ml. After 2 hours of additional incubation, the medium was removed and the cells washed twice in DME medium and subsequently resuspended in the production medium.

The production medium was DME medium supplemented with 0.5% Plasmanate (Cutter Laboratories, Inc., Berkeley, California.) The initial temperature was 37°C. After one hour, the production temperature was shifted to 30°C. After 24 hours, the supernatant was collected and fresh medium added. At 48 hours, again the medium was collected and the microcarriers were discarded. Collected culture fluids were centrifuged at 2000 rpm for 10 minutes and the supernatant frozen at -70°C until assayed for beta-interferon.

Interferon Assay

Interferon production by FS-4 cells was measured by assay for the inhibition of virus-induced cytopathic effect (CPE) as described by Havell and Vilcek, supra. Samples were assayed in duplicate using 96-well microplates. One hundred microliters (ml) of medium (DME supplemented with 2% FCS) was added to each well. Serial two-fold dilutions of prediluted samples were performed in each row of twelve wells. To each well 5×10^4 FS-4 cells in 100 ul of growth medium was added. Thereafter the

plates were incubated for 24 hours at 37°C. Then cells were challenged with 10,000 PFU of VSV (Indiana strain) per well. After challenge, the plates were incubated until the control wells, to which no interferon was added, showed total cell destruction (from 48-72 hours). The plates were scored microscopically and the highest dilution of the sample showing 50% destruction of cells was considered the end-point. An internal standard calibrated against International Standards GO23-901-527 and GO23-902-527 (obtained from National Institutes of Health, Bethesda, MD) was included with each assay.

Cell Detachment by High pH Trypsinization

FS-4 cells grown to confluence on microcarriers were washed extensively and resuspended in 0.2% trypsin solution in 20 mM HEPES buffered at various pH's. After an exposure time of three minutes, a profound change in cell morphology was observed. In Figure 1, cells are shown before trypsinization (Figure 1A), after trypsinization at pH 7.4 (Figure 1B) and after trypsinization at pH 8.6. With increasing pH of the trypsin solution, cells changed from an elongated polarized shape to a more retracted, round form. The retraction of the elongated shape did not occur at the pH range typically used in cell culture (7.4 - 7.0) even after fifteen minutes trypsinization.

To determine whether cells trypsinized at a high pH could attach to microcarriers and grow

normally, FS-4 cells grown to confluence on roller bottles were trypsinized at pH 9.0 and subsequently inoculated into microcarrier culture. Cells so treated grew normally to confluence (data not shown). Thus, short exposure to a high pH did not appear to have any observable detrimental effect on the growth of FS-4 cells.

FS-4 cells grown in microcarriers were trypsinized at various pH values above 7.0 to determine the optimum range of pH for detachment. After treatment, cells could be detached from microcarriers by mild mechanical manipulation, such as repeated pipetting. However, at the culture volume used in this study, 100 to 500 ml, it was more convenient to remove cells by passing the trypsinized microcarrier suspension through a small conduit or through a column packed glass beads as described above. To quantify the effect of pH on cell detachment, confluent cells on microcarriers were treated with a 0.2% solution of trypsin at different pH values and subsequently passed through the packed glass-bead column. As shown in Figure 2, cell detachment by trypsinization improved progressively with increasing pH. Moreover, cells detached by high pH trypsinization reattached to microcarriers and remained viable. At pH of 7.8 and above detachment ranged from about 65 to 95%. Based upon these results, trypsinization was carried out at a pH ranging from 8.4 to 8.8 in further experiments. With a longer exposure (fifteen minutes) to trypsin solution, FS-4 cells were successfully detached by trypsinization at pH 8.2 and recultivated.

Serial Propagation of FS-4 Cells on Microcarriers

Employing the high pH trypsinization to detach cells from microcarriers, FS-4 cells were serially propagated in microcarrier culture. The results of this experiment are summarized in Table 1. A multiplication ratio of three to four was used. Multiplication ratio is the ratio of the total surface area available for cell growth in the newly inoculated culture to that of the seed microcarrier culture. Thus, a 100 ml culture at 5 g/l microcarrier concentration used to inoculate a 400 ml culture at the same microcarrier concentration yields a multiplication ratio of four. The resulting 400 ml culture contains of 1.5 g of new microcarriers and 0.5 g of carried-over used microcarriers.

A microcarrier suspension was inoculated with FS-4 cells grown in roller bottles. At the end of the period of exponential cell growth, 100 ml of the culture were withdrawn and trypsinized at pH 8.6 as described above. About 90% of cells were detached from microcarriers. The detached cells, along with cells remaining on microcarriers, were inoculated into a 400 ml culture (Culture II in Table 2). After cell attachment, a sample was withdrawn for cell enumeration. The resulting cell concentration in the 400 ml culture was 3.4×10^5 cells/ml. There was no apparent loss of cells. The cultures were further incubated until cell concentration reached nearly 10^6 cells/ml before 130 ml were withdrawn for the inoculation of a subsequent culture (Culture III

in Table 1). A 95% cell detachment was achieved. The volume of the third culture in series was also 400 ml and the resulting initial cell concentration was 3.3×10^5 cells/ml. Throughout the cultivation, the medium used was DME medium supplemented with 5% FCS. The kinetics of cell growth are shown in Figure 3. Arrows indicate trypsinization at high pH and inoculation into a new culture.

Table 2
Serial Propagation of FS-4 Cells
on Microcarriers

Culture	I	II	III
Cell concentration at inoculation	3.0×10^5	3.4×10^5	3.3×10^5
Final cell concentration	1.4×10^6	9.6×10^5	9.1×10^5
No. of cells used for subsequent inoculation	1.4×10^7	1.25×10^7	
No. of cells detached	1.28×10^7	1.18×10^8	
% of cells detached	91	95	

Differentiation of the Effect of pH and Trypsin Activity

a. Lack of Improved Cell Detachment with Increased Trypsin Concentration

Brief exposure of FS-4 cells to a 0.2% trypsin solution at pH 8.4-9.0 resulted in morphological changes which allowed cells to be detached from microcarriers and used for subsequent inoculation.

The proteolytic activity of trypsin increases as the pH shifts from acidic (below pH 7) to basic (above pH 7). However, it is unlikely that the effect of high pH on cell detachment is a result of increased trypsin activity. Increasing trypsin concentration three-fold (0.6% trypsin) at normal pH failed to induce the morphological changes which should precede cell detachment. And, as shown in Figure 2, although detachment was improved slightly at the increased trypsin concentration, the effect was not as pronounced as at high pH.

The results shown in Figure 2 do not indicate the viability of cells after detachment from microcarriers. Cells detached after morphological changes are observed to reattach to microcarriers upon ensuing reinoculation. In contrast, most cells detached at pH 7.4 were unable to reattach either to the microcarriers or to Petri dishes.

b. Insensitivity of Trypsin Activity to pH (7.4-9.0)

The proteolytic activity of trypsin solution was assessed at different pH with a chromogenic substrate, Azocoll. As shown in Figure 4, the proteolytic activity of the enzyme did not change much over the tested pH range. Activity increased with increasing pH to a maximum at pH 7.9-8.2. Further increase in pH resulted in a decrease on the proteolytic activity. The increase in the proteolytic activity coincided with the observed improvement in cell detachment shown in Figure 2. The subsequent reduction in proteolytic activity at pH

higher than 8.4, however, did not impede cell detachment. Furthermore, the difference of proteolytic activity over the tested pH range was too small to account for the effect on cell detachment.

The insensitivity of trypsin activity has been reported by Northrop and Kunitz, J. Gen. Physiol. 16, 295-321 (1932). With crystalline trypsin, they found that trypsin activity was optimal at a pH between 7.5 and 9.0; within this optimal range the activity of trypsin showed little variation.

c. Effect of High pH after Trypsinization

To further differentiate the effect of pH from that of the trypsin activity, cells were subjected to trypsin and pH treatment separately. A culture of FS-4 cells was grown to approximate confluence and 4.8×10^7 cells on 250 mg of microcarriers were used for each experiment. Trypsinization and cell detachment were performed as described above except for the following modifications. Trypsinization was carried out in 50 ml centrifuge tubes instead of in chromatographic columns. After thorough washing with PBS, cells were exposed to trypsin solutions. In control cultures, the pH values of the trypsin solution were 8.7 and 7.2, respectively with no subsequent incubation period. Trypsinization was allowed to proceed at room temperature for five minutes after which microcarriers were resuspended in 50 ml of PBS and then passed through the packed column of glass beads. In the other two cases, the trypsinization was also carried out at pH 7.2 for five minutes but, after trypsinization, cells were

incubated in buffered solutions at different pH values. Before incubation, the microcarriers were suspended in 50 ml of PBS and centrifuged at 200 x g for one minute. The supernatant was discarded and the microcarriers were mixed with the HEPES buffers containing 50 mg/l of soybean trypsin inhibitor (Sigma Chemical Co.). The composition of the HEPES buffer was the same as that used for trypsin solution. In one case, the pH of the HEPES buffer was 8.7 and the other 7.2. After incubation in the buffer solution at room temperature for five minutes, the supernatant was withdrawn, the microcarriers resuspended in 50 ml PBS and subsequently passed through the packed glass bead column.

Table 3
Effect of pH after Trypsinization
on Cell Detachment

pH During Trypsinization	pH During Subsequent Incubation	Total Number of Cells Detached	% of Cells Detached
8.7	no subsequent incubation	4.40×10^7	91
7.2	no subsequent incubation	1.26×10^7	27
7.2	8.7	3.80×10^7	78
7.2	7.2	1.46×10^7	31

The detached cells were enumerated in a hematocytometer. As shown in Table 2, more than 90% of cells were detached from microcarriers when trypsinization was carried out at a high pH. In contrast, less than 30% of cells were detached at pH 7.2. After the removal of trypsin, further incubation at a physiological pH had little effect on cell detachment, whereas incubation at pH 8.7 facilitated cell detachment significantly. These results indicate that once binding between cells and the microcarrier surface is loosened by trypsinization, incubation at a high pH can facilitate cell detachment from microcarriers.

Furthermore, improved cell detachment appears to be due to a mechanism other than an increased proteolytic activity at high pH.

Serial Propagation and Product Formation on Microcarriers

A 500 ml culture was inoculated with roller bottle-grown FS-4 cells at a concentration of 7 x 10^4 cells/ml. A high multiplication ratio was achieved by the use of microcarriers having a median diameter 40% larger than the microcarriers routinely used in this laboratory (270 micron median diameter instead of 190 micron), and the use of DME/F-12 (50:50) mixture supplemented with 5% FCS as the medium. Confluent cells from this culture (40 mls of culture; 1.35 x 10^6 cells/ml) were then trypsinized at pH 8.5 and subsequently detached to inoculate a second culture. In the second culture, cells grew normally until reaching a confluent concentration of 1.30 x 10^6 cells/ml. An increase in cell number of about two-hundred fold was achieved in a two stage culture. The kinetics of cell growth are shown in Figure 5. The arrow indicates trypsinization at high pH and reinoculation.

To determine whether cells propagated in this manner were capable of product formation, FS-4 cells from this serially propagated culture were induced to produce Beta-interferon by the superinduction procedure of Giard et al., described above. A control culture was inoculated with cells grown in roller bottles. The production medium was

replenished with fresh medium one day after initiation of the production stage. Beta-interferon produced in the first and second day was assayed as described. As shown in Table 3, interferon productivity of cells serially propagated on microcarriers was comparable to that of a culture inoculated from roller bottles. Thus, FS-4 cells serially propagated on microcarriers using this trypsinization method can grow normally and are capable of interferon production.

Table 4
Beta-Interferon Production by FS-4 Cells Grown on Microcarriers

	Interferon Titer (units/ml)		
	Inoculated from Roller Bottles	Inoculated from Microcarriers	
		Run 1	Run 2
0-24 hr	24,000	24,000	15,000
24-48 hr	11,000	9,600	7,600

To test if this subculture procedure is applicable to other cell types, monkey kidney epithelial cells (Vero) were tested. These cells are commonly used for vaccine production. A culture was inoculated at a concentration of 3.0×10^5 cells/ml and grown to confluence. The confluent cell

concentration was 3.8×10^6 cells/ml. Forty ml of the confluent culture were trypsinized at pH 9.0 with the procedure described above and used for the inoculation of a 500 ml culture at a cell density of 3.1×10^5 cells/ml. The inoculum cell concentration of the resulting daughter culture was 3.1×10^5 cells/ml. As shown in Figure 6, Vero cells thus cultivated grew very well with no appreciable lag phase or decrease in growth rate. After cells grew to confluence, they were infected with vesicular stomatitis virus (VSV) to test the affect of direct inoculation on virus production. The results are shown in Figure 7. The production period lasted for about fifteen hours. The titer of virus obtained was comparable to that reported in the literature, Giard et al., supra.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

CLAIMS

1. A method of detaching anchorage-dependent cells from a positively-charged substrate, comprising treating the cell-laden substrate with a solution of proteolytic enzyme at a pH of about 7.8 to about 10.0 and then, subjecting the treated substrate to a force sufficient to produce shear and thereby detach the cells from the substrate.

2. A method of detaching anchorage-dependent cells from a positively-charged microcarrier, comprising treating cell-laden microcarrier with a solution of proteolytic enzyme at a pH of about 7.8 to about 10.0 and then, subjecting the treated microcarriers to a force sufficient to produce shear and thereby detach the cells from the microcarrier.

3. A method of Claim 2 wherein the microcarriers are positively-charged polydextran beads.

4. A method of Claim 2 wherein the proteolytic agent is an enzyme selected from the group consisting of trypsin, pronase, collagenase and proteinase K, or a mixture of two or more of these enzymes.

5. A method of Claim 2 wherein the pH is from about 8.2 to about 9.0.

6. A method of Claim 2 wherein the step of subjecting the microcarriers to shear force comprises passing the microcarriers through a column of glass beads.

7. A method of Claim 2 wherein the step of subjecting the microcarriers to shear force comprises passing the microcarriers through a small conduit.

8. A method of serially cultivating anchorage-dependent cells on positively-charged microcarriers, comprising the steps of:

a. culturing anchorage-dependent cells on positively-charged microcarriers suspended in a growth medium to a desired growth stage;

b. thereafter separating the cell-laden microcarriers from the growth medium;

c. treating the cell-laden microcarriers with a solution of a proteolytic enzyme at a pH of about 7.8 to about 10.0;

d. subjecting the treated microcarriers to a force sufficient to produce shear and thereby detach the cells from the microcarriers;

e. inoculating the detached cells into a second growth medium in which fresh positively-charged microcarriers are suspended; and

f. culturing the cells in the second growth medium to a desired growth stage.

9. A method of producing anchorage-dependent cell growth-by-products comprising the steps of:

a. culturing anchorage-dependent cells on positively-charged microcarriers suspended in a growth medium to a desired growth stage;

b. thereafter separating the cell-laden microcarriers from the growth medium;

c. treating the cell-laden microcarriers with a solution of a proteolytic enzyme at a pH of about 7.8 to about 10.0;

d. subjecting the treated microcarriers to a force sufficietn to produce shear and thereby detach the cells from the microcarriers;

e. inoculating the detached cells into a second growth medium in which positively-charged microcarriers are suspended;

f. culturing the cells in the second growth medium under conditions conducive to the production of cell growth-by-products; and

g. harvesting the cell growth-by-products.

10. A method of Claim 9 wherein the cell growth-by-product is a hormone.

11. A method of Claim 9 wherein the cell growth-by-product is a virus.

12. A method of Claim 9 wherein the cell growth-by-product is lymphokine.

FIG.1A

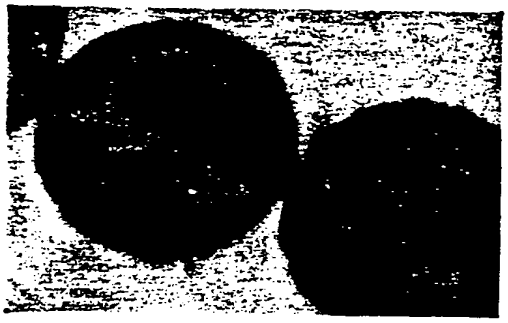


FIG.1B

FIG.1C

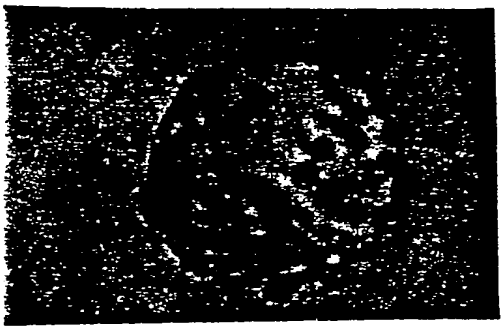


FIG.2

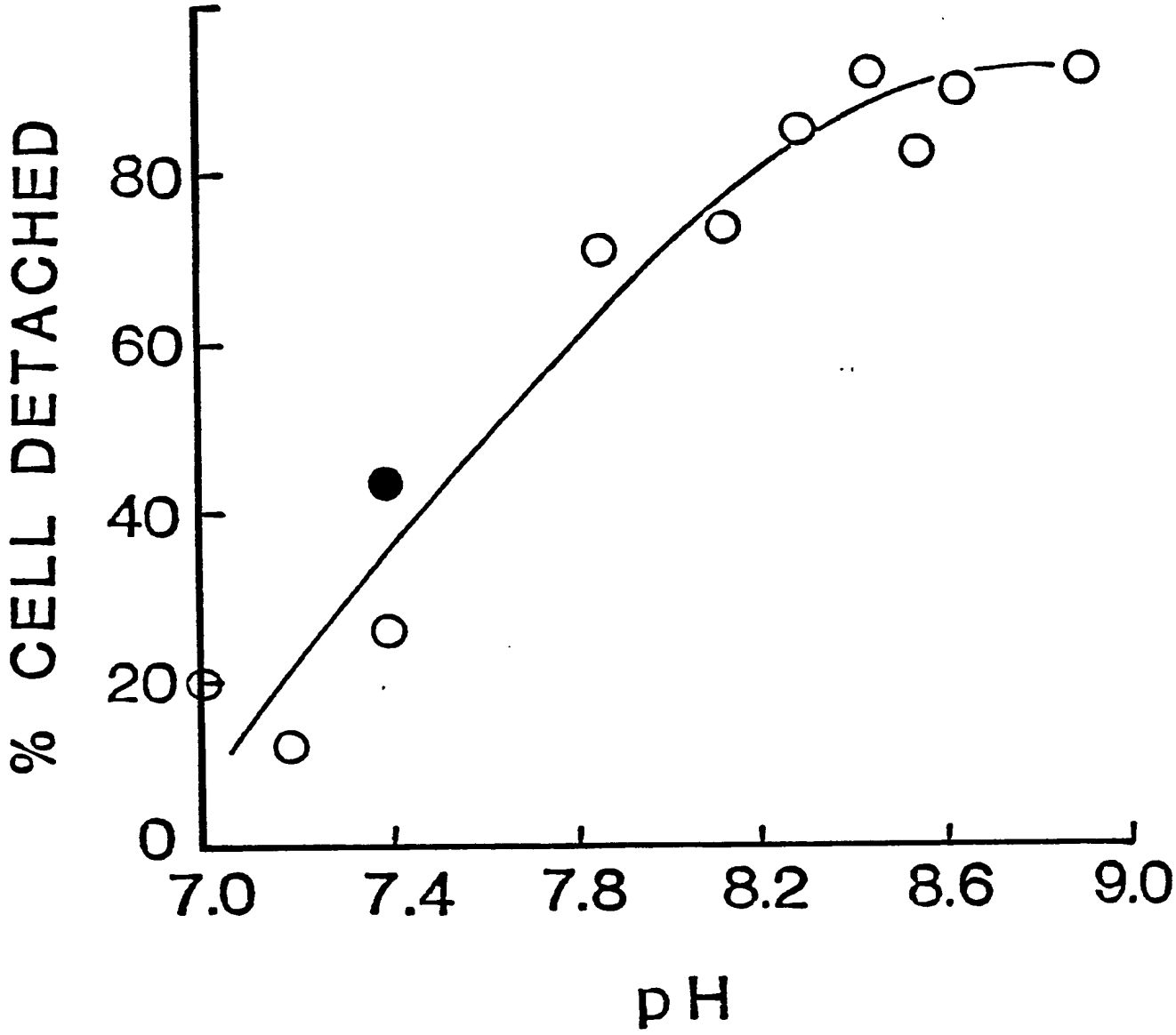


FIG.3

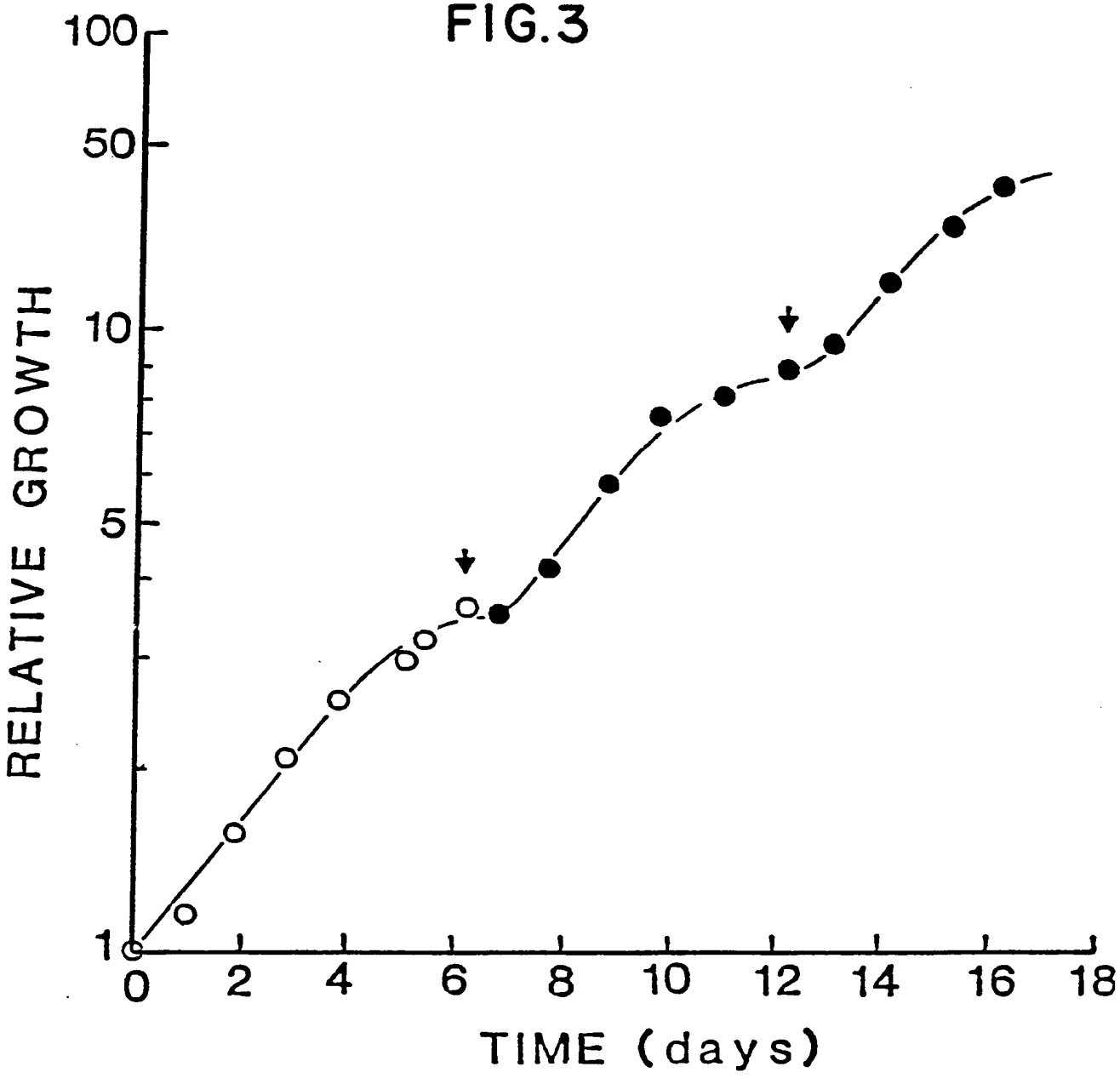


FIG. 4

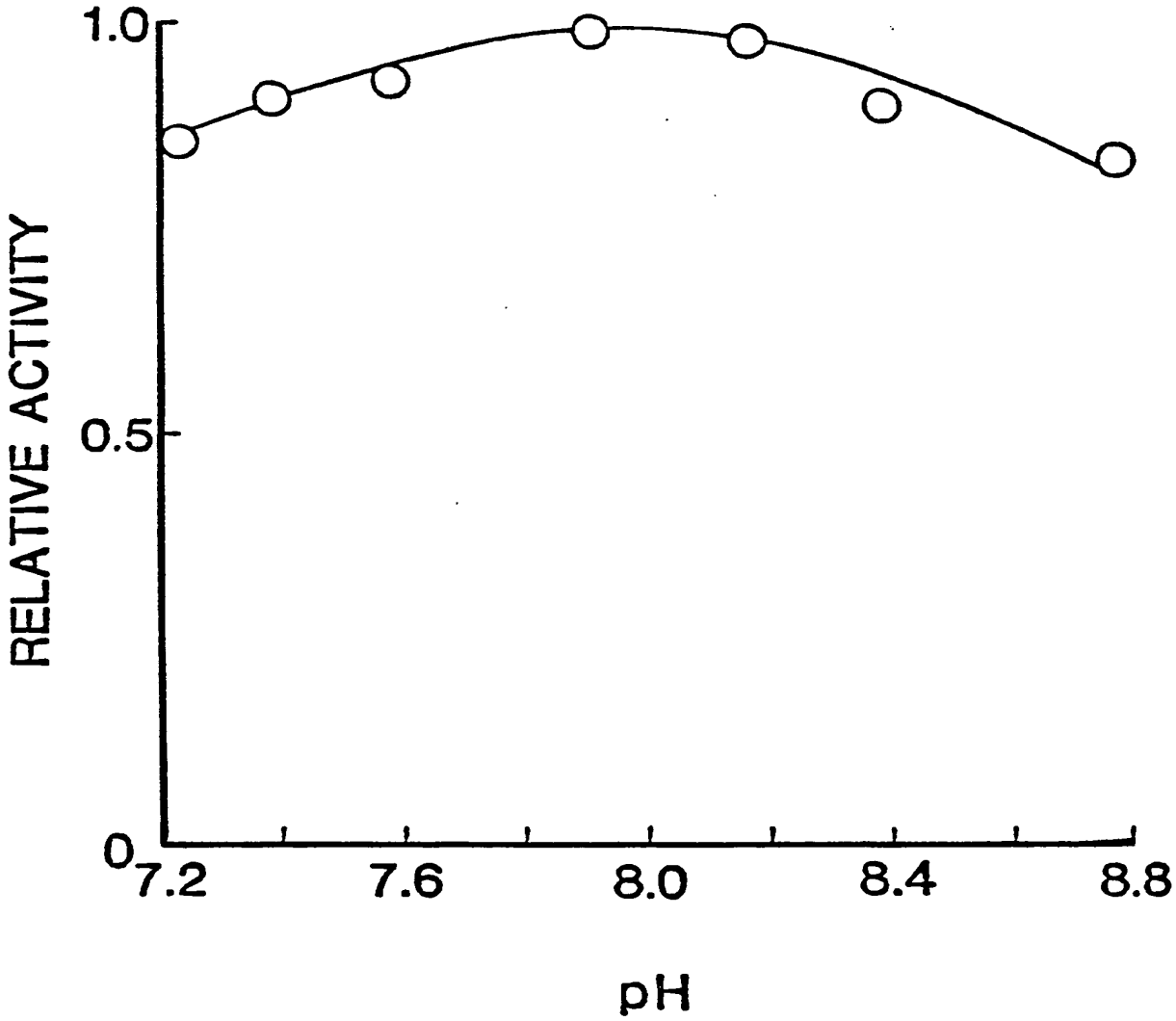


FIG. 5

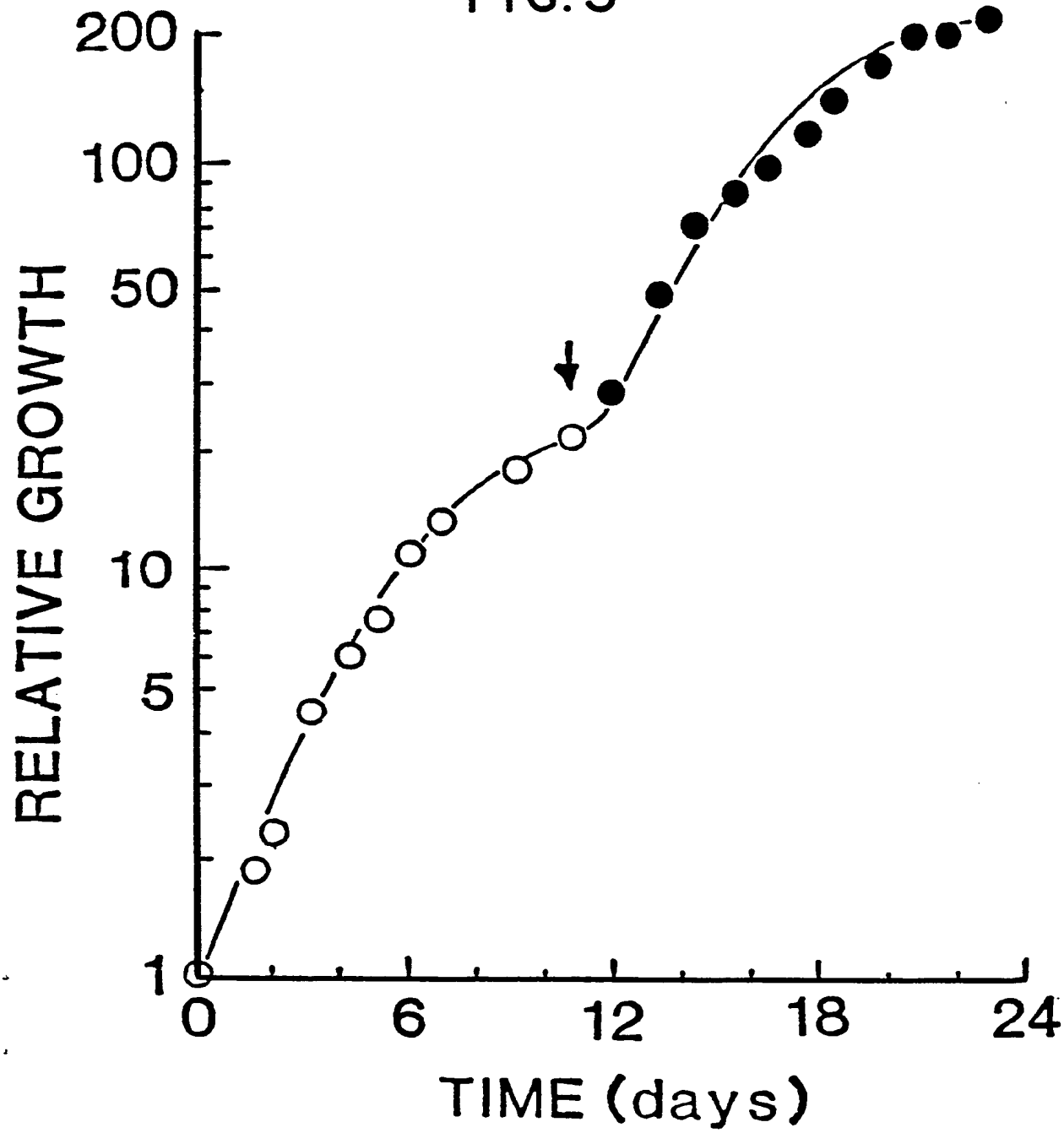


FIG.6

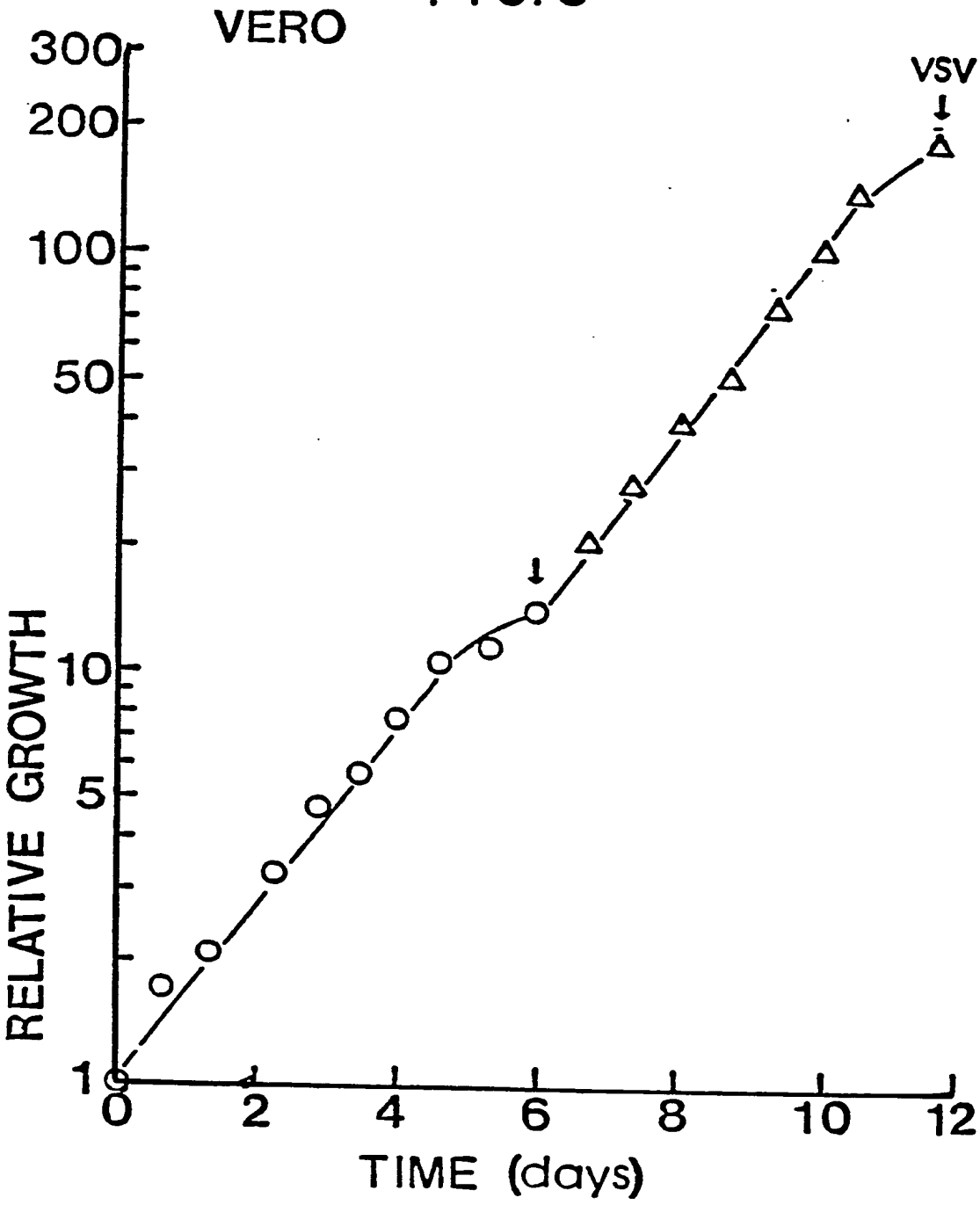
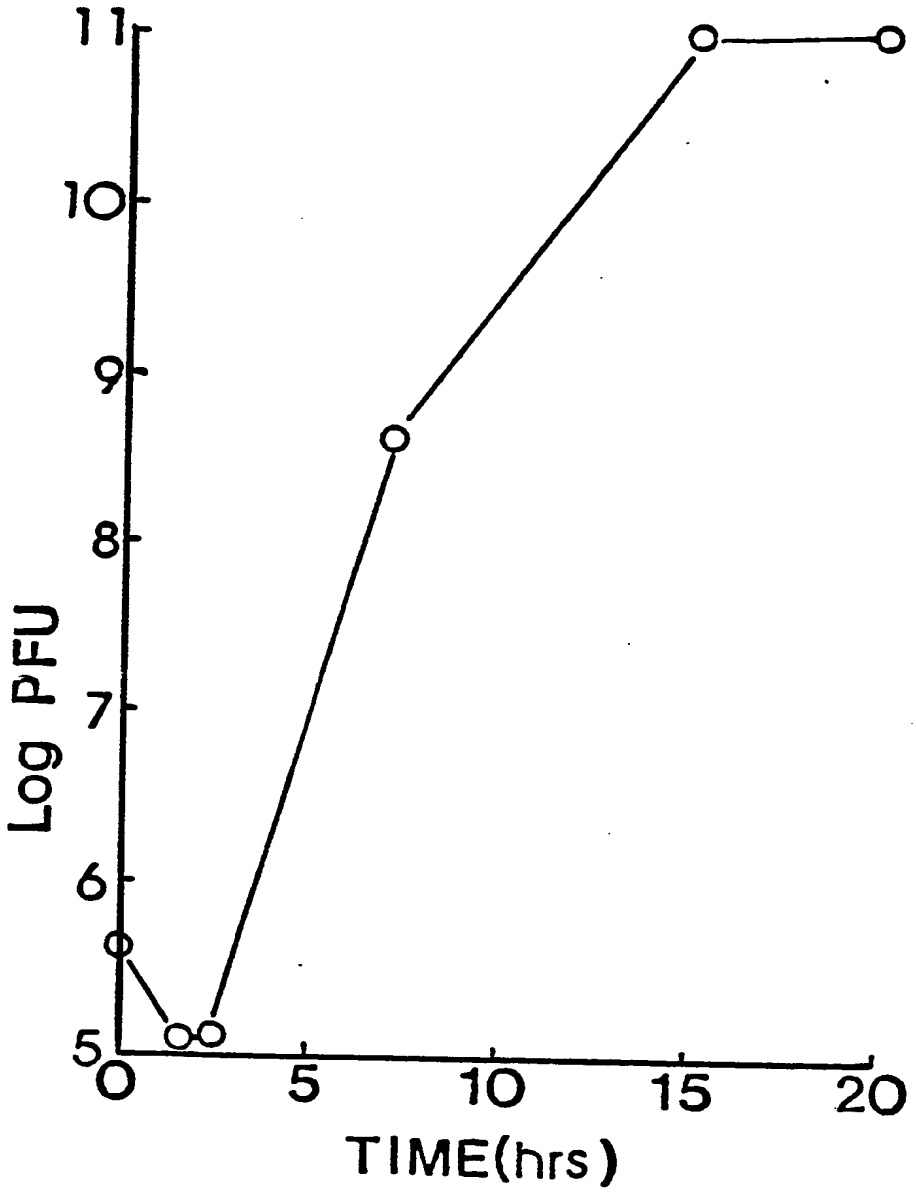

VERO
VSV
300
200
100
50
10
5
1
RELATIVE GROWTH
0
2
4
6
8
10
12
TIME (days)

FIG. 7



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 85/01615

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 5/02; C 12 P 21/00; C 12 N 7/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	GB, A, 2059991 (PHARMACIA FINE CHEMICALS AB) 29 April 1981, see page 1, lines 27-37; page 3, lines 6-9; example 2; claims 1,6 --	1-12
A	US, A, 4189534 (D.W. LEVINE et al.) 19 February 1980, see column 3, line 42 - column 4, line 9; column 7, example 2; claims 7,13-16 (cited in the application) --	1-12
A	Biotechnology-Bioengineering, volume 23, 1981, John Wiley & Sons, Inc. C.L. Crespi et al.:"Microcarrier Culture: Applications in Biologicals Production and Cell Biology", see page 2675, lines 7-17; page 2676, lines 25-36; page 2678, lines 2-18,23-41; page 2685, lines 5-25 --	1-12
A	Biotechnology-Bioengineering, volume 23, 1981, John Wiley & Sons, Inc. C.L. Crespi et al.:"Continuous cell propagation using low-charge micro-carriers", see page 983, line 12 - page 984, line 11; page 989, lines 10-18 -------	1-12

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th December 1985	10 JAN. 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	G.L.M. Kruydenberg

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/US 85/01615 (SA 10528)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 06/01/86

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
GB-A- 2059991	29/04/81	FR-A,B	2464994	20/03/81
		DE-A-	3033885	02/04/81
		JP-A-	56051981	09/05/81
		SE-A-	7907573	13/03/81
US-A- 4189534	19/02/80	BE-A-	860721	01/03/78
		NL-A-	7712202	16/05/78
		FR-A,B	2370792	09/06/78
		DE-A,B,C	2749989	18/05/78
		GB-A-	1535150	06/12/78
		JP-A-	53062889	05/06/78
		AU-A-	3049177	28/06/79
		CA-A-	1063050	25/09/79
		CH-A-	615947	29/02/80
		AU-B-	508398	20/03/80
		JP-A-	56099789	11/08/81
		JP-A-	56099790	11/08/81
		SE-A-	7712700	11/05/78
		SE-A-	8006993	07/10/80
		SE-B-	417108	23/02/81
		DE-C-	2759579	25/08/83
		US-A-	4293654	06/10/81